Isothiocyanates. XLVII.*
Characterization of macromolecular polyisothiocyanates

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The ability of cellulose isothiocyanates to bind low molecular amines, thiols, and proteins by covalent bonds from diluted aqueous solutions were characterized. The influence of basic parameters such as the concentration of reactants, temperature, pH of the medium, reaction time, and stability of the linkage on the reaction course were investigated with cellulose isothiocyanates using [^{14}C] glycine, 2-mercaptoethanol, and [^{131}I] albumin. The mentioned nucleophiles can be used for characterizing the binding properties of macromolecular polyisothiocyanates as well as their suitability for preparation of immobilized enzymes.

Была характеризована способность изотиоцианатов целлюлозы образовать ковалентные связи с низкомолекулярными аминами, тиолами и протеинами из разбавленных водяных растворов. С помощью [^{14}C] глицина, 2-меркаптоэтанола и [^{131}I] альбумина, исследовались у изотиоцианатов целлюлозы основные параметры реакции, такие как температура, концентрация реагирующих веществ, pH среды, реакционное время и стабильность связи. Приведенные нуклеофильные реагенты можно использовать для характеризации как связывающих свойств высокомолекулярных полиизотиоцианатов, так и возможности приготовления фиксированных ферментов.

In the process of interaction of insoluble macromolecular compounds with solutions of low and high molecular compounds, physicochemical phenomena such as adsorption, inclusion, ionic and covalent bonds, hydrogen bonds, and hydrophobic interactions are involved. These phenomena can be employed in preparation of immobilized enzymes on solid supports and in binding of other low and high molecular compounds. Considering the design of suitable macromolecular carriers it is desirable to know which of the mentioned phenomena is the dominating one. The use of macromolecular polyisothiocyanates in the preparation of immobilized enzymes by binding their amino groups with the –NCS groups of the matrix is one of the potential principles for further realization of biochemical reactors. The properties of such a system are given by the properties of the used skeleton. At present, it is not possible to predict these properties without determining the enzymatic properties of the

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reactor. This procedure is time-consuming and it is not suitable for a rapid judgement on the properties of the prepared polymers.

The present study concerns the elaboration of rapid methods for characterization of the binding properties of cellulose isothiocyanates using compounds containing $-NH_2$ and $-SH$ groups (glycine, albumin, and 2-mercaptoethanol).

Experimental

Chemicals and instruments

The preparation of cellulose isothiocyanates: *I.* cellulose —O—CH_2—CH_2—NCS, *II.* cellulose —O—CH_2—CO—NH— ⬡ — ⬡ —NH—CS—NH— ⬡ —NCS, *III.* cellulose —O—CH_2—CH_2—NH—CS—NH— ⬡ —NCS, *IV.* cellulose —O—CO—NH—$(CH_2)_5$—NCS is described in [1]. [2 – ^{14}C] glycine (50 Ci/mol) was obtained from the Institute for Research, Production, and Uses of Radioisotopes, Prague; [^{131}I] human serum albumin (the initial radioactivity 50 μCi/mg) was purchased from the Institute of Nuclear Research, Radioisotope Production and Distribution Centre, Otwock, Poland.

The incorporated radioactivity in the washed samples collected on filter papers Whatman (30 mm diameter) was counted using a Methane flow counter 41 T, Frieseke und Hoepfner GmbH (West Germany). Spectrophotometric measurements were carried out on a Specord UV VIS (Zeiss, Jena) spectrophotometer. pH Measurements of buffers accurate to ±0.01 pH unit were obtained with a Radelkis (Hungary) pH meter. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) (Aldrich Chem. Co.), 2-mercaptoethanol (Sigma, Co.), the other chemicals, the solvents, and the components of buffer were of anal. grade.

Reactions of cellulose isothiocyanates with [^{14}C] glycine and [^{131}I] albumin

Samples of cellulose isothiocyanates (20 mg) in 0.1 M phosphate buffer of pH 8.0 (1 ml) with an addition of [^{131}I] albumin (20 μg) and [^{14}C] glycine (3 μg) were maintained at 25°C under stirring for 2 and 24 hrs, respectively. After this time the suspension was diluted with a fivefold amount of the used buffer containing unlabelled albumin (1 mg/ml) and glycine (10 mg/ml), respectively. Then it was suspended three times in the same buffer, centrifuged, and filtered. The incorporated radioactivity was counted after drying and in some instances converted into absolute amount of the bound substance.

Reactions of cellulose isothiocyanates with 2-mercaptoethanol

A series of cellulose isothiocyanate samples (150 mg) in 0.2 M phosphate buffer of pH 8.0 (2 ml) with an addition of thiol (13 mM) was maintained under stirring at 25°C. Aliquot parts of the supernatants were withdrawn at suitable time intervals and transferred into 0.1 M TRIS buffer of pH 7.2 containing EDTA (20 mM). DTNB [2] was added up to the resulting concentration 475 μM and after 5 min the sample was diluted with a fourfold volume of methanol. The absorbance was measured at 412 nm and the loss of thiol was calculated from the calibration curve or from the value of the molar absorption coefficient $\varepsilon_{412} = 1.35 \times 10^4\ M^{-1}\ cm^{-1}$.

Results and discussion

The amount of covalently bound glycine and albumin was established on the basis of the radioactivity incorporated in the polymer that could not be removed by a repeated washing with buffers containing unlabelled albumin or glycine. The reaction conditions were based upon the previous results on the reaction kinetics of polyisothiocyanates with glycine and albumin in dependence on the concentration of reactants and pH values of the reaction mixtures.

Fig. 1 shows the reaction kinetics of [^{131}I] albumin with the cellulose isothiocyanate *I* which had the –NCS group coupled to the cellulose skeleton through the ethylene group. Under the given conditions, the reaction was practically completed in 2 hrs. At this time, the total amount of the bound albumin was a function of the initial concentration of albumin in the solution (Fig. 2, curve *1*) and of the amount of polyisothiocyanate in a volume unit of the reaction mixture. With increasing amount of polyisothiocyanate in a volume unit of the solution the total amount of albumin bound to the polymer increased, however, the amount of the bound albumin related to a weight unit of the polymer decreased (Fig. 2, curve *2*). The mentioned data may be significant in optimalization of conditions for immobilization of enzymes with this type of carriers because the total enzymatic capacity of the system will depend on the amount of the bound enzyme (up to a certain value). In such systems beside chemical reaction also other interactions might play an essential role, therefore, as a next task we determined to what an extent did the above results represent the actual amount of albumin bound covalently to the polyisothiocyanate. At present, such a characterization of covalently binding systems is considered to be indispensable for estimation of their properties [3].

When polyisothiocyanate *II* was repeatedly washed after the reaction with [^{131}I] albumin (with a solution containing unlabelled albumin) it was found that a part of albumin bound from the solution could be removed which was evident from the elution diagrams (Fig. 3). The elution profile with this polyisothiocyanate and the one with the unsubstituted cellulose differed significantly. In the case of polyisothiocyanate it was possible to remove as much as 35% of the total bound albumin by a repeated washing (Fig. 3*b*). The remaining part was bound so strongly that it could not be released even by a treatment with alkali hydroxide solutions. According to our knowledge on the reactions of isothiocyanates with various –SH and $-NH_2$ compounds [4—7], this 65% albumin remaining after treatment with hydroxides represented albumin bound covalently *via* $-NH_2$ groups. Generally the reaction of amino groups with isothiocyanates is conditioned by the presence of unprotonated amino groups. Their concentration is determined by the preceding dissociation reaction [4]. The used

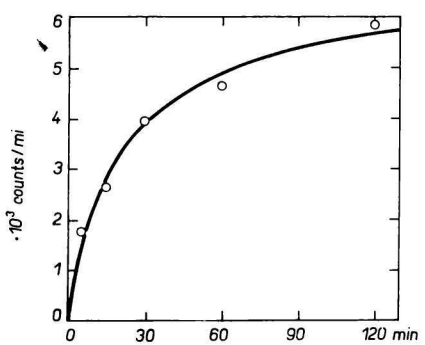

Fig. 1. Reaction kinetics of [^{131}I] albumin (87 μg/ml) with polyisothiocyanate *I* (20 mg/ml) in 0.1 M-K_2HPO_4 of pH 8.0 at 25°C.

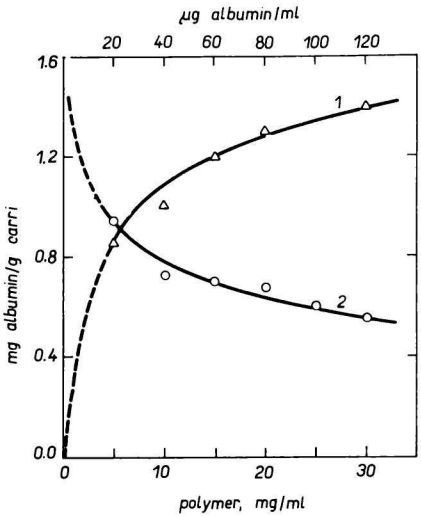

Fig. 2. Concentration effect of the reactants on the amount of [^{131}I] albumin bound to polyisothiocyanate *II*.
0.1 M-K_2HPO_4, pH 8.0, 25°C, reaction time 2 hrs.
1. Albumin (concentration of *II* 10 mg/ml);
2. polymer *II* (concentration of albumin 20 μg/ml).

preparatives of [^{131}I] albumin were found to contain 0.5 mole of free –SH groups (determined with DTNB) per mole of albumin, however, the linkage of –SH group with the –NCS group was considerably unstable towards alkali hydrolysis [7].

The results obtained in the study of the reaction of polyisothiocyanates with [^{14}C] glycine are presented in Fig. 4 (time dependence) and Fig. 5 (concentration dependence).

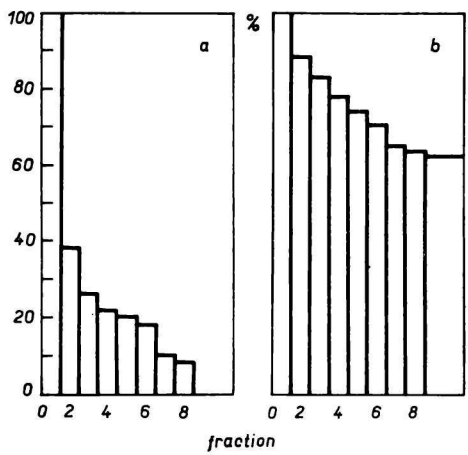

Fig. 3. Elution diagram for [^{131}I] albumin bound to polyisothiocyanate *II* (*b*) and unsubstituted cellulose (*a*).
100 mg of the polymer and 600 μg of [^{131}I] albumin in 100 ml reaction mixture of pH 7.0; 1 hr at 25°C. The sediment of the reaction mixture was repeatedly washed with 10 ml 0.15 M-NaCl (fractions *1—6*) and with 10 ml 0.1 M-Na_2CO_3 (fractions *7*, *8*). Radioactivity was counted in aliquots of supernatants and in the fibrillous material.

The amount of bound 2-mercaptoethanol was established from the concentration decrease in the reaction mixture at the presence of the polyisothiocyanate suspension under continuous stirring. The decrease of thiol concentration was determined in a supernatant of the reaction mixture by *Ellman* reagent [2] at the chosen reaction time (1 hr). The lower was the initial thiol concentration in the solution, the more significant appeared to be the spontaneous oxidation. However, when the thiol concentration was above 5 mM, the rate of the spontaneous oxidation was unimportant.

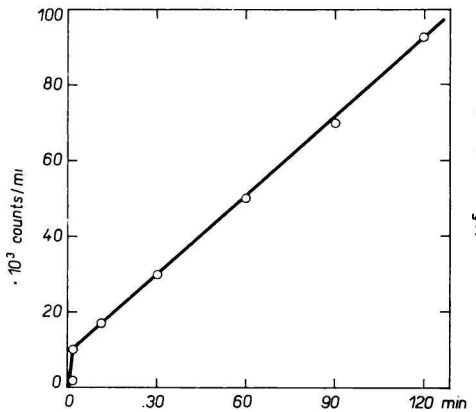

Fig. 4. Reaction kinetics of [^{14}C] glycine (2.96 μg/ml) with polyisothiocyanate *II* (20 mg/ml) in 0.1 M-K_2HPO_4 of pH 8.0 at 25°C.

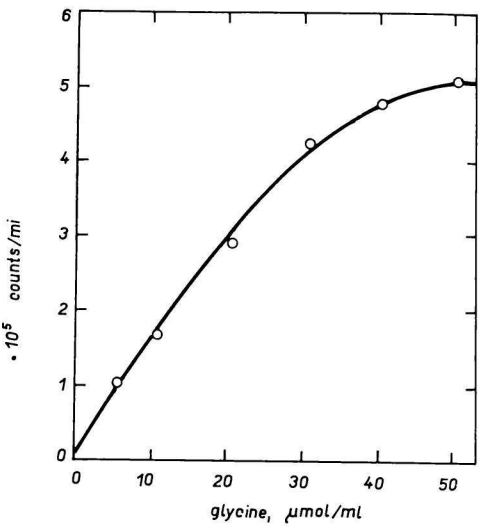

Fig. 5. Amount of [^{14}C] glycine bound to polyisothiocyanate *II* (20 mg/ml) in dependence on the initial concentration of glycine in the reaction mixture.
0.1 M-K_2HPO_4, pH 8.0, 25°C, reaction time 4 hrs.

It was found that the amount of reacted 2-mercaptoethanol was a function of pH of the reaction mixture with the optimum in the range of pH 8.0—10.0 (Fig. 6). The reaction rate of polyisothiocyanates with the thiol compounds was manyfold higher than those with the low or high molecular amino compounds (Figs. 4 and 5) which was in accordance with our previous experience [4—9]. When the pH value of the reaction mixture was below 6, the amount of the bound 2-mercaptoethanol was negligible because the isothiocyanates reacted only with the anionic form of a thiol group [5, 6]. The sharp decrease of the reacted amount of thiol in an alkali medium (above pH 10) could be explained by inactivation of the –NCS groups of polyisothiocyanates by a reaction with OH^- ions of the medium resulting in the formation of thiocarbamates and their subsequent decomposition to primary amines [10]. A similar shape of the pH dependence was found also in the reaction with [^{14}C] glycine. Fig. 7 shows the amount of the bound 2-mercaptoethanol in dependence on the amount of polyisothiocyanate in a unit volume of the solution.

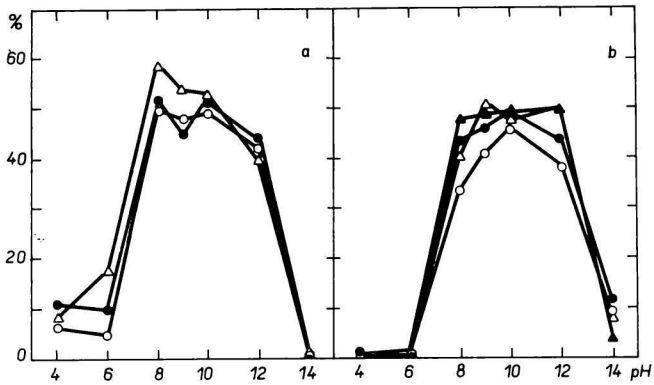

Fig. 6. pH Dependence of the amount of 2-mercaptoethanol bound by polyisothiocyanates from 0.2 M phosphate buffers containing 20 mM EDTA and 13 mM 2-mercaptoethanol at 25°C.
a) Polyisothiocyanate *III* (75 mg/ml): ○ 15 min; ● 30 min; △ 60 min.
b) Polyisothiocyanate *IV* (25 mg/ml): ○ 1 min; ● 5 min; △ 10 min; ▲ 15 min.

The amounts of [^{14}C] glycine, 2-mercaptoethanol, and [^{131}I] albumin coupled to the substances *I*—*IV* are presented in Table 1. There is an obvious correlation ($r = 0.98$) between the binding capacity determined by [^{14}C] glycine and 2-mercaptoethanol. Such a correlation was not found with [^{131}I] albumin. In the reaction of low molecular compounds with cellulose isothiocyanates the shape of the pH dependence of the amount of the $-NH_2$ and –SH groups reacted in a certain time-interval was similar to the ionization curve of the corresponding reacting groups. In the reaction of albumin with cellulose isothiocyanates the shape of the mentioned dependence was entirely different. The evidence mentioned indicates that in the interaction of cellulose isothiocyanates with proteins, other factors, such as steric effects and ionic hydrogen bonds may play an important role.

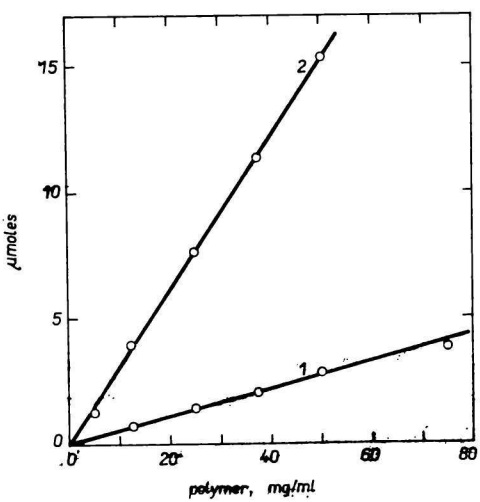

Fig. 7. Amount of 2-mercaptoethanol bound to polyisothiocyanates *III* (*1*) and *IV* (*2*) in dependence on concentration of the polymer suspension.
0.2 M phosphate buffer of pH 9.0 with 20 mM EDTA, the initial concentration of 2-mercaptoethanol 13 mM, 25°C.

Table I

Binding capacity of cellulose isothiocyanates *I—IV*

Derivative	[^{14}C] Glycine	2-Mercaptoethanol	[^{131}I] Albumin
	μequiv. –NCS/g		mg protein/g
I	48.0	155	0.1
II	1.0	27	1.9
III	16.7	55	0.1
IV	21.5	65	5.3

The amount of the bound thiol was a linear function of the amount of the used polymer in a volume unit of the solution. When using cellulose isothiocyanates in practice for binding biomolecules of protein and peptide nature, it can be presumed that depending on the pH value of the used medium, either the thiol groups only or both the thiol and the free amino groups will react with the –NCS groups similarly as in the reactions with low molecular isothiocyanates in solutions or in the interactions with cells [11].

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